POLYCLONAL ANTI-PEPTIDE ANTIBODIES AGAINST BOTH ACTIVATED AND UNACTIVATED FORMS OF THE HUMAN GLUCOCORTICOID RECEPTOR

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Name of Candidate: Delia Ching Tang
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Delia Ching Tang
Department of Pharmacology
Uniformed Services University
of the Health Sciences

ABSTRACT

Title of Thesis: **Polyclonal antipeptide antibodies against both activated and unactivated forms of the human glucocorticoid receptor**

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The human glucocorticoid receptor (hGR) is a member of a superfamily of ligand-responsive transcription factors. The hGR is comprised of a unique amino-terminus (or hypervariable region), a central DNA-binding domain and a carboxyl terminus (or ligand-binding domain). Regulation of hGR expression is multifactorial, involving transcriptional, post-transcriptional, and post- translational components. In order to study this regulation in detail, we sought to develop specific anti-hGR antibodies. On the basis of hydrophilicity/hydrophobicity ratios of the protein molecule, as well as secondary structure predictions, a peptide corresponding to amino acid residues Cys_{245}-Thr_{272} within the amino terminus was synthesized and conjugated to keyhole limpet hemacyanin (KLH). Anti-hGR antisera were generated in three New Zealand White rabbits. Immunoabsorption of [^{3}H]triamcinolone acetonide ([^{3}H]TA)-labeled hGR showed all three antisera recognized the native form of the protein. In each case, the concentration of antisera required to absorb

50% of the receptor was less than that for polyclonal antisera previously raised against the purified, intact receptor protein. Preincubation of the antisera with saturating concentrations of the immunizing peptide completely blocked the immunoabsorption of the [^{3}H]TA-hGR hormone-receptor complex. Incubation of the ligand-receptor complex with the antipeptide antisera caused a discrete increase in the sedimentation coefficient of both activated and unactivated [^{3}H]TA-hGR complexes on sucrose gradients. In addition, a major immunoreactive protein with a molecular weight of 92 kDa, corresponding to the molecular weight of the hGR, was identified by the antisera by Western blot analysis, indicating the antisera also react with the denatured form of the hGR. These combined results demonstrate the usefulness of the antisera in the biochemical characterization of the native and denatured hGR.

POLYCLONAL ANTIPEPTIDE ANTIBODIES AGAINST BOTH ACTIVATED AND UNACTIVATED FORMS OF THE HUMAN GLUCOCORTICOID RECEPTOR

by

Delia Ching Tang

Thesis submitted to the Faculty of the Department of Pharmacology Graduated Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Master of Science 1993

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ABBREVATIONS

Buffers

HBSS	Hanks Balanced Saline Solution 48 mg/L Na_2HPO_4, 97.7 mg/L $MgSO_4$, 60 mg/L KH_2PO_4, 400 mg/L KCl, 140 mg/L $CaCl_2$, 1 g/L glucose
Homogenization Buffer	10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid, 1 mM EDTA, 10 mM NaCl, 0.5 mM dithiothreitol; pH 7.6
2x Laemmli Sample Buffer	0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethonal
Transfer Buffer	0.025 M Tris base, 0.192 M glycine, 20% methanol
TBS	20 mM Tris, 0.5 M NaCl; pH 7.5
HEDG Buffer	10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid, 1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol; pH 7.6

INTRODUCTION

The glucocorticoid receptor (GR) is a member of a superfamily of ligand-inducible transcription factors, which includes the receptors for the adrenal steroids (glucocorticoids and mineralocorticoids), sex steroids (estrogen, progesterone, and androgen), thyroid hormone, all *trans*- retinoic acid, and vitamin D_3 (for review: Green et al., 1986; Evans, 1988). The cDNAs for all major steroid hormone receptors have been cloned and sequenced. They were related in structure and led to definition of an even larger family of regulatory proteins. This superfamily was shown to include oncogenes such as v-*erb*-A (Sap et al., 1986; Green et al., 1986). In addition, this superfamily includes a number of orphan receptors whose ligands have not been identified and whose functions are not known: estrogen-receptor related protein 1 (ERR-1) (Giguere, 1988). Each member of the family appears to have similar structure and act through a similar mechanism.

I. The action of steroid hormones and receptors

The overall pathway by which glucocorticoids act is largely known. Glucocorticoids enter the target cell by passive diffusion. The GR is a soluble cytosolic protein and binds specifically to the ligand glucocorticoid. This

interaction facilitates an activation (or transformation) of the steroid-receptor complex, resulting in the stabilization of the interaction between steroid and protein, the translocation of GR into the nucleus, and the acquisition of the ability of the complex to bind to specific DNA sequences (glucocorticoid response elements; GREs), thereby altering the transcription rate of hormonally responsive genes. Ultimately, these changes in transcription are expressed in new protein synthesis and modification of cell function. Thus, the receptor appears to be a direct signal transducer, converting the signal information of the circulating hormone into specific changes in gene transcription by interacting specifically with both the hormone and the gene (Yamamoto, 1985).

II. Functional domains of the GR

Biochemical analysis of crude cytosolic GR or analysis of affinity-labeled GR by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a single polypeptide species with a molecular weight of 90-100 kDa. Limited proteolysis of the GR (Wrange and Gustafsson, 1978; Carlstedt-Duke et al., 1982, 1987), using a variety of proteolytic enzymes, and insertion and deletion mutagenesis (Giguere et al., 1986; Kumar et al., 1986; Danielsen et al., 1987; Hollenberg et al., 1987; Rusconi and Yamamoto, 1987) suggested that the GR is structurally organized into three

distinct functional domains. The availability of cloned GR cDNA has enabled the definition of these functional domains at the genetic level as well.

The C-terminal part of the GR is responsible for ligand binding (Giguere et al., 1986) and contains regions important for nuclear localization (Picard and Yamamoto, 1987), and binding of heat shock protein 90 (hsp90) (Denis et al., 1988, Pratt et al., 1988). The middle part is short and highly conserved. This domain is responsible for the specific binding to GREs (Green and Chambon, 1987; Rusconi and Yamamoto, 1987; Freedman et al., 1988; Dahlman et al., 1989). The function of the N-terminal part of GR is less well understood, but it contains a region involved in transcriptional transactivation (Giguere et al., 1986; Hollenberg and Evans, 1988).

1. Steroid-binding domain

The steroid-binding domain has been defined by proteolysis of the purified GR with trypsin. Mild digestion with trypsin resulted in a proteolytic fragment with Mr=27,000, determined by SDS-PAGE. Sequence analysis of the N-terminus of this fragment showed that it corresponds to the C-terminal part of the protein, with the domain border at residue 499 for human GR (hGR), and residue 518 for rat GR (rGR) (Carlstedt-Duke et al., 1987). Analysis of deletion

mutants of the GR cDNA confirmed the C-terminal localization of the steroid-binding activity. Hollenberg (Hollenberg and Evans, 1988) isolated a GR cDNA clone, which lacked 50 C-terminal residues and did not bind steroid. This has been confirmed by deletion of various portions of the open reading frame corresponding to the C-terminus of hGR (Giguere and Evans, 1986) or rGR (Godowski and Yamamoto, 1987). One of the mutant forms of the mouse GR is immunologically reactive but has no hormone-binding activity. Sequence analysis of this mutant showed a point mutation resulting in the substitution of gly for glu at residue 546 (equivalent to rat residue 558 and human residue 540) (Danielsen et al., 1986). This mutation was shown to be sufficient to destroy the steroid-binding activity.

2. DNA-binding domain

The DNA-binding domain of the GR was originally defined by proteolysis with α-chymotrypsin. This digestion results in a proteolytic fragment of Mr=39,000 containing both the steroid-binding and DNA-binding domains. Further digestion of this fragment with trypsin revealed an intact steroid-binding domain (Wrange et al., 1978; Carlstedt-Duke et al., 1987). Amino acid sequence analysis revealed a mixture of N-termini, indicating cleavage by α-chymotrypsin at at least two sites within the region of residues 409-413 (Carlstedt-Duke et al., 1987). Thus the functional DNA-

binding domain is contained within residues 410/414-517 (equivalent to hGR residues 390/394-498) (Gustafsson et al., 1987).

This region corresponds to the region of GR cDNA that shows a very high degree of homology with other steroid receptors as well as with the oncogene product v-*erb*-*A* (Hollenberg and Evans, 1985; Loosfeld et al., 1986; Danielsen et al., 1986; Weinberger and Evans, 1985). This region is rich in cysteine, arginine, and lysine, and corresponds to residues 440-505 within the rat GR. Thus, the DNA-binding domain identified by sequence homology lies entirely within the functional DNA-binding domain defined at the protein level.

This region contains two repeated motif (Evans and Hollenberg, 1988) similar to that of the zinc-stabilized DNA binding "fingers" of the 5S rRNA transcription factor TFIIIA (Brown et al., 1985; Miller et al., 1985). The TFIIIA motif contains a pair of cysteines and a pair of histidines which are believed to tetrahedrally co-ordinate a zinc ion (Miller et al., 1985). In contrast, the receptor motifs contain pairs of cysteines which appear to be important for receptor function (Green and Chambon, 1987) and DNA-binding (Kumar and Chambon, 1988). The two zinc atoms, tetrahedrally coordinated by conserved cysteine residues, are required for proper folding and DNA binding (Freedman et al., 1988).

Analysis of a cDNA clone isolated from a nuclear transfer deficient (nt^-) mouse S49 cell mutant revealed a single point mutation within the DNA-binding region resulting in the substitution of his for arg at residue 484 (equivalent to rat residue 496 and human residue 477). This substitution was shown to destroy the DNA-binding capacity of the GR that was otherwise intact (Danielsen et al., 1986).

The highly conserved 70-residue DNA-binding domain mediates specific binding to hormone response elements in DNA (Yamamoto, 1985; Gustafsson, 1987; Evans, 1988; Beato, 1989). Protein fragments containing the glucocorticoid receptor DNA-binding domain expressed in *Escherichia coli* exhibit sequence-specific binding to DNA (Freedman et al., 1988; Dahlman et al., 1989). Most glucocorticoid-regulated genes have short *cis*-acting enhancer-like sequences termed glucocorticoid responsive elements (GREs) in or near their transcription units (Geisse et al., 1982; Jantzen et al., 1987). These GREs are palindromic, one half of the palindrome containing the sequence TGTTCT, and the other TGTACC, with the two halves separated by 3 bp (Klock et al., 1987). Using band-shift assays to examine the interactions of the GRE with receptor, it has been observed that the receptor interacts with its cognate enhancer as a dimer. The DNA binding domain of the glucocorticoid receptor binds first to the TGTTCT half-site of the GRE, and a second molecule binds

subsequently to the TGTACA half-site of the GRE in a cooperative manner (Tsai and O'Malley, 1988). The crystal structure of the glucocorticoid receptor DNA-binding domain complexed with two oligonucleotides, which differ only in their half site spacing by one base pair, has been examined. Crystallographic analysis of these two complexes demonstrated that the spacing between the half-sites is critical for the target sequence's identity (Luisi et al., 1991).

Cloning and sequencing the hGR gene has shown that it is split into nine exons (Encio and Detera-Wadleigh, 1991). One of the introns occurs in the middle of the highly conserved DNA-binding domain inviting speculation that each half of DNA-binding domain may correspond to separate functional sub-domains. Results from Chambon's group show that the two sub-regions CI (N-terminal, first finger) and CII (second finger) appear to be separate domains both involved in DNA binding (Green and Chambon, 1988).

Green and Chambon replaced the DNA-binding domain region of the estrogen receptor (ER)with that of the glucocorticoid receptor and showed that DNA binding specificity had been converted. This showed that the DBD determines the receptor's specificity (Green and Chambon, 1987). Using chimeric ERs in which either the first (CI) or second (CII) 'zinc finger' region was exchanged with that of the GR, it was shown that it is the first 'zinc finger' which

largely determines target gene specificity. The receptor recognition of GRE is analogous to that of the helix-turn-helix DNA binding motif in that the receptor binds to DNA as a dimer with the first 'zinc finger' lying in the major groove recognizing one half of the palindromic GRE. A chimeric estrogen receptor, in which the CI finger is replaced with the corresponding glucocorticoid receptor CI finger region, activates transcription from a reporter gene containing a GRE, but not form a reporter gene containing an ERE (estrogen responsive element). Further, mutagenesis demonstrated that three amino acids (Gly, Ser and Val) located at the C-terminal side of the estrogen receptor CI finger are essential to distinguish an estrogen from a glucocorticoid responsive element (Mader et al., 1989). The three-dimensional structure of the DNA-binding domain (DBD) of the glucocorticoid receptor has been determined by nuclear magnetic resonance spectroscopy and distance geometry. A model of the dimeric complex between the DBD and the glucocorticoid response element is proposed. The model is consistent with previous results indicating that specific amino acid resides of the DBD are involved in protein-DNA and protein-protein interactions (Hard et al., 1990). The protein-DNA interaction is stabilized through non-specific DNA binding and dimer interactions contributed by the second 'zinc finger'.

3. Amino-terminal domain

The steroid- and DNA-binding domains account for approximately half of the protein. The remaining N-terminal half of the protein is a hypervariable region having low homology between receptors of the steroid/thyroid hormone receptor superfamily. The epitopes for most antibodies that have been raised to steroid receptors are located in this hypervariable region (Carlstedt-Duke et al., 1982; Westphal et al., 1982; Green et al., 1984; Sullivan et al., 1986). It has been suggested that this region may contribute to the diversity of specificity at the various receptors. The N-terminal domain is less well defined and may have a modulatory effect on *trans*-activation.

N-terminal deletion of 439 amino acids gives rise to a mutant rat GR that still demonstrates dexamethasone-dependent transcriptional activation. However, N-terminal deletion of 464 amino acids destroys all transcriptional activity of the rat GR. Thus the N-terminus of the minimal unit necessary for transcriptional activation corresponds to the region defined by homology comparisons between receptors. In addition, the interdomain border defined by trypsin [residues 517/518 (Carlstedt-Duke, 1987)] corresponds exactly to the border of the region for transcriptional enhancing

activity defined by deletion mutants [residues 509-525 (Miesfeld and Yamamoto, 1987)].

III. Genomic structure of GR gene

By somatic-cell hybridization, Gehring et al. demonstrated that the hGR gene is located on chromosome 5 (Gehring et al., 1985). The hGR gene contains a total of 9 exons and spans approximately 150 kilobases (Encio et al., 1990). Exon 1 consists of solely of 5'-untranslated sequence, and exon 2 encodes the amino-terminal portion of the receptor, containing the initiator methionine codon, other potential start sites, and all of the amino terminal domain up to the DNA binding domain (Zong et al., 1990). The two putative zinc fingers are separately encoded by two exons, and a total of four exons (exons 5-9) combine to form the steroid-binding domain (Encio et al., 1990). Exon 9 encodes the last few amino acids of ligand binding domain and all of the 3' untranslated region.

IV. Nuclear localization signals

Nuclear accumulation of receptor appears to be mediated by nuclear localization signals, the protein segments thought either to facilitate diffusion and intranuclear retention, or to interact with putative active transport machinery (Picard et al., 1987). Modulation of the

activity of such signals might play a role in the hormone-dependent receptor activation. Using a transient transfection expression assay, and immunofluorescence to determine subcellular distribution of receptor derivatives and β-galactosidase-receptor fusion proteins, two distinct nuclear localization signals, NL1 and NL2, were defined. NL1 maps to a 28 amino acid segment closely associated, but not coincident with the DNA binding domain; NL2 residues within a 256 amino acid region that also includes the hormone binding domain (Picard and Yamamoto, 1987).

V. Activation (transformation) of the GR

Binding of the steroid hormone to the receptor results in a temperature-dependent structural change of the hormone-receptor complex from a form that is recovered in the cytosolic fraction to a form that is recovered in the nucleus. This ligand-mediated change is called activation, or transformation, and it is accompanied in the intact cell by a change in the receptor from a non-DNA-binding to a DNA-binding state (Middlebrook et al., 1975; Antakly and Eisen, 1984; Walter et al., 1985; Welchon et al., 1987; Qi et al., 1990). The receptor also undergoes a ligand-initiated translocation from the cytoplasm to the nucleus (Wikstrom et al., 1987; Picard and Yamamoto, 1987).

Non-activated glucocorticoid receptor found in the cytosol of target cells has a sedimentation coefficient of 8S and a Stokes radius of 7-8 nm, corresponding to an oligomeric structure with a molecular weight of 300,000 (Sherman et al., 1984; Holbrook et al., 1985). Activated complexes have a molecular weight of 100,000 (sedimentation coefficient of 4S, Stokes radius of 5-6 nm) (Sherman et al., 1984; Holbrook et al., 1985).

The large 8S complex can be observed when analysis is performed in the presence of molybdate or other transition metal oxyanions from group VI-A (Leach et al., 1979; Dahlmer et al., 1984). A direct interaction between molybdate and receptor, possibly by formation of bridge(s) between the different components of 8S complex (Wilson et al., 1986), contributes to the ability of molybdate to stabilize the receptor (Grandics et al., 1984; Idziorek et al., 1985; Lustenberger et al., 1985; Hapgood et al., 1987). An endogenous heat-stable, molybdate-like factor in the cytosol has been found to stabilize the glucocorticoid receptor in its untransformed, non-DNA-binding form (Meshinchi et al., 1990). Sodium molybdate mimics the ability of this endogenous factor to stabilize the untransformed receptor. Because of its ability to stabilize the non-activated glucocorticoid receptor, molybdate has been extremely useful in characterization and purification studies.

The subunit composition of the oligomeric non-activated glucocorticoid receptor has not yet been completely elucidated. On the basis of the molecular weight of glucocorticoid receptor complexes calculated from Stokes radii and sedimentation coefficients determined in the absence or presence of molybdate, the non-activated receptor has been suggested to be composed of several subunits (Vedeckis et al., 1983; Raaka et al., 1983), including a single Mr=92,000 steroid-binding unit (Okret et al., 1985) and a dimer of Mr=90,000 non-steroid-binding protein (Housley et al., 1985; Mendel et al., 1986; Denis et al., 1987), which has been identified as heat shock protein 90 (hsp90) (Sanchez et al., 1985; Catelli et al., 1985). The clear correlations, between transcriptional activity and the non-hsp90-containing forms, and between inactivity and heteroligemeric hsp90-containing forms, provide evidence that hsp90 can be a critical factor in maintaining the receptor in a nonfunctional state.

Hsp90 interacts with the ligand binding domain of the glucocorticoid receptor. Amino acids 574-659 are involved in forming a stable receptor-hsp90 complex and the region 632-659 is especially important (Howard et al., 1990; Dalman et al., 1991). The critical region, amino acids 632-659, contains a short proline-containing hydrophobic segment and an adjacent dipole-plus-cysteine motif that is conserved among all of the hsp90-binding receptors in the superfamily.

The second hsp90 contact site is in the region 574-632, which contains the only highly conserved amino acid sequence in the receptor superfamily outside of the DNA-binding domain. The receptor becomes associated with hsp90 during late translation, or immediately at the termination of translation (Dalman et al 1989). In addition, hsp70 and RNA have been found in association with the oligomeric complex (Tsai et al., 1986, Webb et al., 1986), as well as hsp56 (Sanchez, 1990).

Interaction of the ligand-binding domain with hsp90 would interfere with several ligand-binding domain (LBD) and DNA-binding domain (DBD) functions, resulting in the repression of the transcriptional activity of GR. The interaction of the receptor with a receptor associated protein(s) masks the sequences required for DNA binding and transactivation. Upon hormone binding, the protein(s) dissociates from the receptor and the sequences are exposed. (Picard et al., 1988)

When steroid-bound receptors in cell-free lysates are exposed to increased temperature, ionic strength, or pH, the receptors are transformed from a non-DNA-binding state to a DNA-binding form (Schmidt et al., 1982). Receptor activation requires both hormone binding and moderate temperature (20-37^{0}C), and is characterized by hsp90 release and a reduction in the sedimentation rate of the receptor in crude cell

extracts under nondenaturing conditions. Nuclear localization is necessary for receptor activation (Picard et al., 1988).

A model has been described from Yamamoto's lab for signal transduction by the GR (Yamamoto et al., 1988). The newly synthesized receptor folds in such a way as to present only the interaction site for hsp90. The hsp90-receptor interaction results in receptor refolding, which produces the active ligand-binding region but inactivates other receptor functions. Upon hormone binding and incubation at moderate temperature, a conformational change in the ligand-binding domain is triggered, causing release of hsp90, protein refolding, and relief of inactivation. The receptor now assumes its functional conformation, with emergent structures responsible for nuclear localization, specific DNA binding, and transcriptional enhancement and repression.

Hsp90 does not inhibit receptor function solely by steric interference. Rather, it seems to have an active role in signal transduction by interacting with the aporeceptor and determining its ability to assume or maintain an activatable conformation. Hsp90 interaction would therefore have two consequences: i) it would establish or stabilize the conformation of the active ligand-binding domain; and ii) it would inactivate other functional domains in a manner that would readily be reversed upon hormone binding and hsp90 release.

Other heat shock proteins, such as hsp70, also display unfoldase activity (Rothman et al., 1986; Pelham, 1988). In addition, proteins related to hsp90 and hsp70 appear to mediate unfolding functions that are required for proper assembly of complex multiprotein structures (Pelham, 1986; Ellis, 1987; Hemmingsen et al., 1988).

VI. Objectives

Despite the success of mutational analysis in defining the domain structure and function of the steroid hormone receptor superfamily, detailed analysis of the GR protein still requires a variety immunological tools. A number of polyclonal and monoclonal antibodies specific for the GR have been produced in recent years (Harrison et al., 1984; Gametchu et al., 1984; Okret et al., 1984; Westphal et al., 1984; Harmon et al., 1984; Eisen et al., 1988; Urda et al., 1989). Most of these were prepared against highly purified preparations of receptor protein. The anti-human GR antisera, AC40, and 884 used in our laboratory were raised against 50% pure GR isolated from the human lymphoid cell line IM-9 (Harmon et al., 1984; Eisen et al., 1988). Consequently, these antisera contain antibodies against other non-GR human proteins which complicate the interpretation of Western blots. Previously, our laboratory attempted to obtain better antibodies by isolation of monoclonal antibody

producing hybridoma cells after immunization of mice with partially purified IM-9 GR, as well as a fusion protein of β-galactosidase and the immunogenic domain of the human GR expressed in *E. coli*. Unfortunately, none of these approaches proved successful. In addition, polyclonal and monoclonal antibodies raised against the rat GR fail to cross-react with the human GR (Stevens et al., 1981). Therefore, a highly specific anti-human GR antibody with little background in Western blots was desired.

The availability of amino acid sequence derived from the DNA sequence has enabled synthetic peptides to be designed for use in preparing epitope-specific antibodies. Studies from our laboratory showed that highly selective antibodies to the human GR can be produced by immunization of rabbits with synthetic peptides that reflect the primary sequence of the human GR (Urda et al., 1989). During screening of a GR cDNA library with a rabbit polyclonal antiserum to the purified glucocorticoid receptor, several immunopositive clones were isolated (Hollenberg et al., 1985). These clones all contained inserts expressing antigenic determinants of the human glucocorticoid receptor, indicating that they contained a common sequence which presumably delimits the major immunogenic domain of the receptor. These clones were sequenced and the primary sequence of the immunogenic domain of GR deduced. In this study, the derived peptide sequence, which is highly

epitotic, was used to raise new antibodies. By characterizing the interaction of these antisera with the human glucocorticoid receptor obtained from lymphoid cells we determined that they recognize both the activated and unactivated forms of glucocorticoid receptor. It should be possible to employ these antibodies to examine the regulation of the hGR.

MATERIALS AND METHODS

Cell Culture

Human lymphoid IM-9 cells were grown in RPMI 1640 (Roswell Park Memorial Institute; Hazelton, Lenexa, KS) in the presence of 10% heat-inactivated fetal bovine serum (Hazelton, Lenexa, KS), and glutamine as previously described (Norman et al., 1977). Cells were grown as stationary suspensions in a humidified atmosphere of 95% air and 5% CO_2. Cells were maintained in logarithmic phase at a density between $1x10^5$ to $2x10^6$ cells/ml. Cell count was obtained with a Coulter Counter (Coulter Electronics, Hialeah, Fla).

Cytosol Preparation

IM-9 cells were grown to approximately $1x10^6$ cells/ml, harvested by centrifugation (800 x*g*) at 4^0C and washed twice in HBSS (Hanks Balanced Saline Solution, 48 mg/L Na_2HPO_4, 97.7 mg/L $MgSO_4$, 60 mg/L KH_2PO_4, 400 mg/L KCl, 140 mg/L $CaCl_2$, 1 g/L glucose). Unless otherwise indicated, all further work was performed at 0-4°C. The final cell pellet was resuspended in an equal volume of homogenization buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid, 1 mM EDTA, 10 mM NaCl, 0.5 mM dithiothreitol; pH 7.6], and immediately homogenized with 15 strokes of a Ten Broeck ground glass homogenizer. Following centrifugation at 150,000x*g* for 90 minutes, the supernatant (cytosol) was immediately adjusted to contain 10% glycerol and 0.1 M NaCl. Cytosols prepared in

this manner were either used immediately or quick frozen in dry ice and stored in liquid nitrogen until use. Frozen cytosols contain 100% of receptor binding activity for up to 3 months (Harmon et al., 1984).

Immunization of rabbits

Three Female New Zealand White rabbits (709, 710, 711) were immunized at 4 weeks intervals with 200 µl of synthetic peptide Cys_{245}-Thr_{272} conjugated to KLH (keyhole limpet hemocyanin) (Multiple Peptide Systems, San Diego, CA) at multiple(20) intradermal sites in the back. The first two immunizations of each rabbit were performed in complete Freund's adjuvant. Subsequent immunizations were performed with incomplete Freund's adjuvant. Animals were bled before the first immunization and 10 days after each immunization, and sera were tested for antibody activity. Positive sera were detected in three rabbits. After the fourth immunization, the sera were collected in large amounts (30 ml) each week until 200 ml had been obtained.

Determination of antibody activities

IM-9 cell cytosol (1.0 ml) was labeled with 10 µl $5x10^{-6}$M [^{3}H]TA for 2 hours at 4^{0}C. Free ligand was removed with dextran coated charcoal. Aliquots of the supernatant ([^{3}H]TA labeled IM-9 cytosol, 50 µl) were incubated with 0.1 µl, 1.0 µl, or 10 µl of immune serum or AC40 for 4 hours,

followed by addition of 100 µl (10%, wt/vol) Pansorbin (BRL, Rockville, MD) suspended in HEDG buffer containing 0.1 M NaCl. After incubation for 30 minutes, with vortexing every 5 minutes, conjugates were pelleted by centrifugation at 12,000x*g* and washed three times in the same buffer. The final pellets were resuspended in 1.0 ml buffer and bound [^{3}H]TA quantified by liquid scintillation counting using a Beckman LS-7800 liquid scintillation spectrometer programmed for dpm conversion. For peptide inhibition studies, assay mixtures were prepared under the same conditions, except that 2 µl 10^{-6} M synthetic peptide was added at the same time as the sera.

Western Blot Analysis

SDS-PAGE was performed as described by Laemmli (Laemmli 1970) in 8% polyacrylamide separating gels with a 3% polyacrylamide stacking gel. Molecular weights were determined from standard curves constructed from the mobilities of the prestained proteins: phosphorylase b (Mr=101,000), BSA (Mr=74,000), ovalbumin (Mr=50,000), and carbonic anhydrase (Mr=33,000). Samples were prepared in 2x Laemmli sample buffer and heated at 95°C for 5 minutes. Electrophoresis was performed at constant voltage (30V) overnight in a Hoefer SE600 series electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA). Directly following SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membranes using a Transfor Electrophoresis Unit (TE series; Hoefer Scientific

Instruments, San Francisco, CA). The transfer was performed at 0.5A for 4 hours in buffer containing 0.025 M Tris base, 0.192 M glycine and 20% methanol. Following transfer and brief staining with Ponceau S Solution (Sigma, St. Louis, MO), the membrane was cut into strips which were incubated for 1 hour with TBS (20 mM Tris, 0.5 M NaCl; pH 7.5) containing 2% non-fat dry milk. Blocked strips were then incubated overnight at 4^0C in 10 ml of TBS containing 2% non-fat dry milk and a 1:100, 1:500, or 1:1000 dilution of preimmune sera, polyclonal anti-human GR antisera AC40, or test sera from rabbits 709, 710, and 711. The strips were washed sequentially (for 15 min each) with TBS containing first 0.5% Tween 20, then 0.5 M NaCl, and finally 0.1% BSA, and then incubated for 1 hr with ^{125}I-protein A (0.0025 mCi, 30Ci/mg; Amersham, Arlington Heights, IL) in TBS containing 0.1% BSA. Following sequential 15 min washes in TBS containing first 0.1% BSA, then 0.5% Tween 20, and finally 0.5 M NaCl, filters were air-dried and exposed to Kodak XAR-5 X-ray film (X-OMAT AR5, Kodak, Rochester NY) at -70^0C for three days.

Sucrose Density Gradient Analysis

100 µl [^{3}H]TA-GR complex were diluted with 100 µl HEDG buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid-1 mM EDTA-0.5 mM dithiothreitol, 10% glycerol; pH 7.6) containing either 0.5 M NaCl and no molybdate, or 0.05 M NaCl

and 20 mM molybdate. 10 µl of 1:10 diluted preimmune serum, or immune serum 710, or immune serum 710 with 2 µl 10^{-6} M synthetic peptide Cys_{245}-Thr_{272} were incubated with the 200 µl diluted [^{3}H]TA-GR complex for at least four hours. Samples (200 µl) were layered onto 4.8 ml 5% to 20% linear sucrose gradients in the corresponding buffers. Gradients were subjected to centrifugation in a Beckman SW50.1 rotor at 200,000x*g* for 18 hours at 4^{0}C and then fractionated into 0.2 ml fractions using a Buchler Auto Densi-Flow IIC gradient fractionater. All gradients contained [^{14}C]-labeled-bovine serum albumin as an internal standard. Approximate sedimentation coefficients were determined as described by Martin and Ames (Martin et al., 1961).

RESULTS

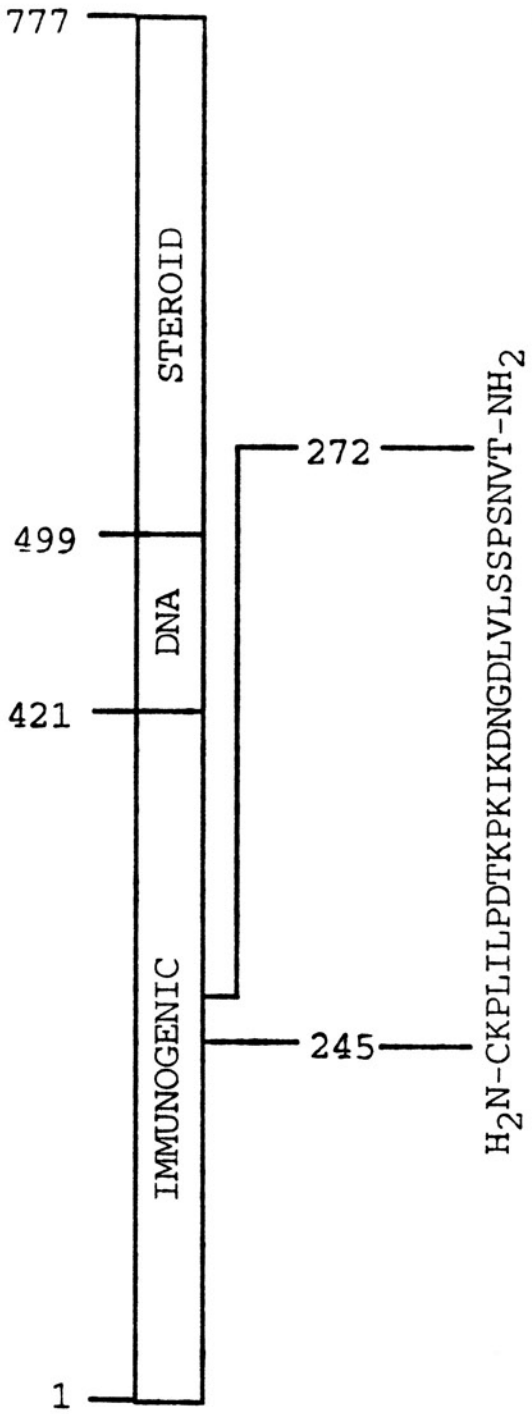
I. Preparation of anti-peptide anti-human glucocorticoid receptor antisera.

1. Production of anti-peptide antisera.

According to hydrophicility/hydrophobicity ratios, as well as secondary structure predictions, a synthetic 28-amino acids peptide (Figure 1), corresponding to residues Cys_{245}-Thr_{272} of the human glucocorticoid receptor, was used to raise epitope-specific anti-GR antibodies. This peptide corresponds to a sequence beginning with the C-terminal cysteine within the immunogenic amino-terminus. The peptide corresponds to a region of the immunogenic domain whose primary sequence has been derived from immunopositive clones selected from a human cDNA library with polyclonal antisera raised against purified glucocorticoid receptor. This peptide is hydrophic and rich in proline residues, which are generally considered to disrupt α-helical regions. The peptide was synthesized with its carboxyl terminal residue amidated, and was conjugated through the N-terminal cysteine to keyhole limpet hemocyanin (KLH) by Multiple Peptide Systems. This conjugated preparation was used to immunize three female New Zealand white rabbits (709, 710, 711). Rabbits were bled before the first immunization to collect preimmune serum and ten days

Figure 1. Schematic Diagram of the Human GR and the Location of Cys_{245}-Thr_{272}.

The locations of the immunogenic, DNA binding, and steroid binding domains of the human GR are illustrated. The peptide corresponding to amino acid residues Cys_{245}-Thr_{272}, located in the immunogenic domain, was synthesized and its carboxyl terminal residue amidated. In addition, this peptide is rich in prolines.


777
STEROID
499
DNA
421
IMMUNOGENIC
1
272
245
H2N-CKPLILPDTKPKIKDNGDLVLSSPSNVT-NH2

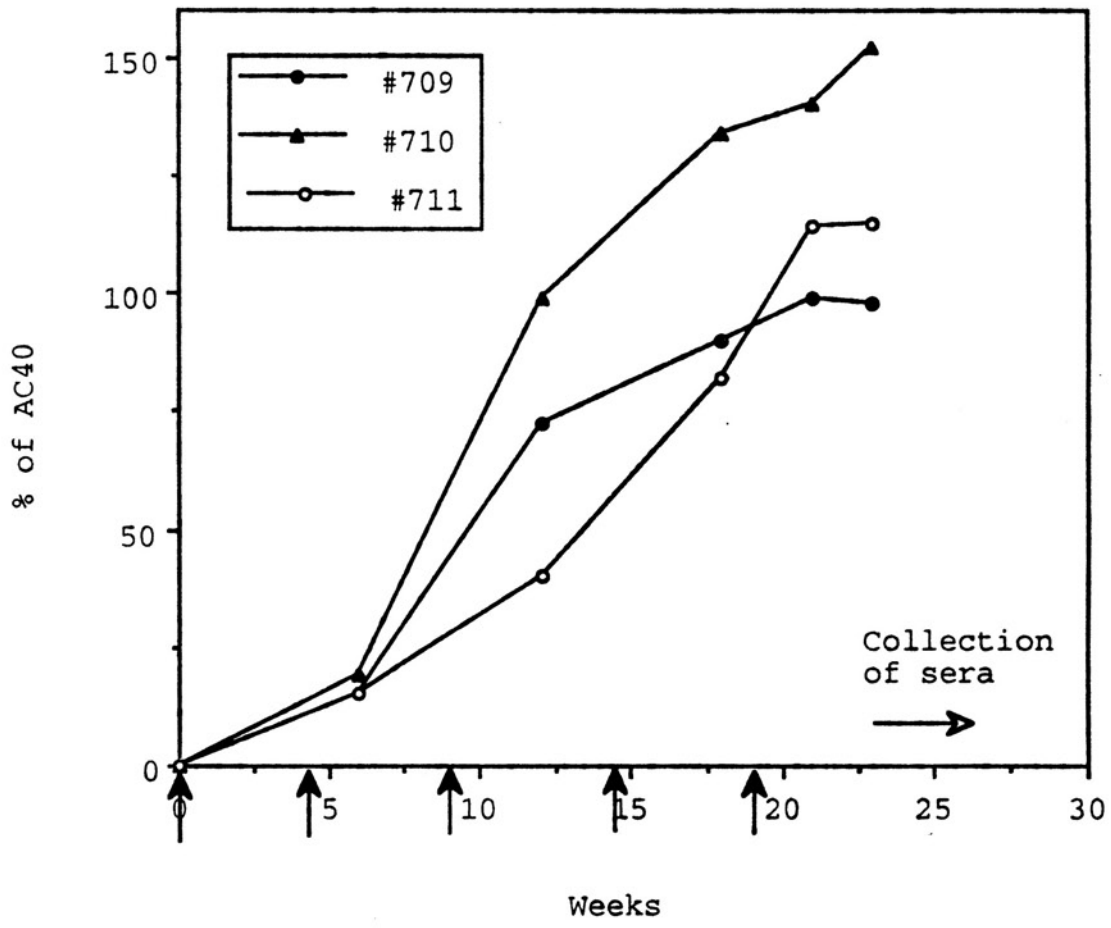
after each subsequent immunization. The ability of newly produced sera to interact with native hGR was determined by precipitation of [^{3}H]triamcinolone acetonide ([^{3}H]TA) labeled receptor-antibody complexes with formalin-fixed *Staphylococcus aureus* membranes containing Protein A. After the fourth immunization sera were collected from each immunized animal and kept at -70^{0}C for later analysis.

2. Time course of immunization.

The presence of antisera containing anti-peptide antibodies reacting with the hGR was confirmed by Western blot analysis. A component of 92 kDa, identical in size to the IM-9 cell glucocorticoid receptor was clearly identified with all three immune sera (data not shown). In contrast, preimmune sera showed no bands of 92 kDa. Positive serum from each immunized rabbit was then analyzed for immunoreactivity with the steroid-bound form of native GR (Figure 2). [^{3}H]TA bound human GR (IM-9) was incubated with either preimmune serum, or 10 µl of each tested serum, or the anti-hGR antibody AC40. Immunocomplexes were adsorbed with immobilized protein A (Pansobin). Measurement of radioactivity in the pellets showed immune specific adsorption of [^{3}H]TA-GR complexes. The activities of the anti-peptide antisera were expressed as the percentage of counts adsorbed by the polyclonal antibody AC40, which was raised against the authentic purified human GR protein (Eisen et al., 1988). In

Figure.2 Time Course of Immunization.

Three female New Zealand white rabbits 709(l), 710(s), and 711(m) were immunized at four week intervals at multiple intradermal sites with 200 µl of synthetic peptide conjugated to keyhole limpet hemacyanin (KLH). Rabbits were bled before the first immunization to collect pre-immune serum and ten days after each subsequent immunization. These sera were tested for antibody activity by incubating 50 µl of [^{3}H]TA-labeled IM-9 cytosol with 10 µl of serum for 4 hours, followed by adsorption of the [^{3}H]TA-hGR-antibody complexes with protein A. The activities of the anti-peptide antisera are expressed as the percentage of counts adsorbed by another antibody (AC40) raised against the intact purified hGR. Vertical arrows indicate the time of immunization. Horizontal arrow indicates the production of antisera. After three or four immunizations all three rabbits produced high titer anti-hGR antibody. The results clearly show that all three antisera recognized the native form of the protein.


150
100
50
0
% of AC40
#709
#710
#711
Collection
of sera
0
5
10
15
20
25
30
Weeks

each rabbit tested, no anti-receptor antibody activity was detected in preimmune serum, and only low activity in serum taken after the primary and secondary immunizations. In the case of rabbits 709 and 710, significant activity was detected after the third immunization, and a further increase in activity was observed after the fourth immunization (Figure 2). In the case of rabbit 711, significant activity was observed after the fourth immunization. After three or four immunizations, all three rabbits produced high titer anti-hGR antibodies.

II. Reactions of anti-peptide antibodies with native forms of human GR.

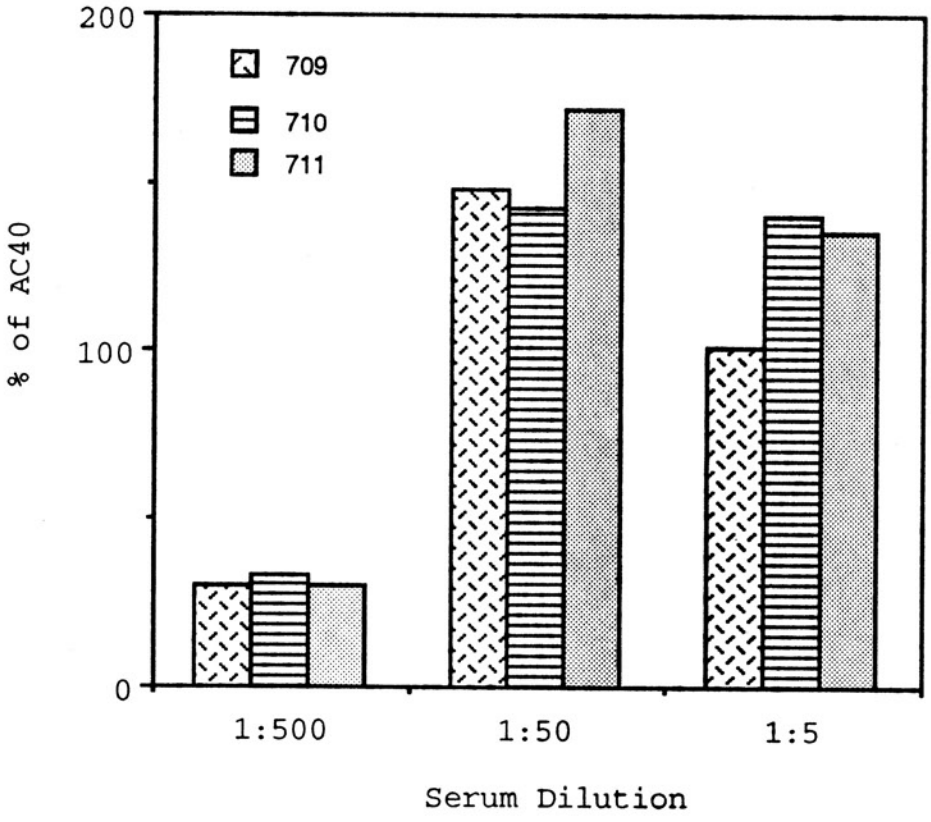
The ability of antisera to interact with the native forms of the hGR was determined in several ways.

1. Titration analysis of anti-hGR activity.

To examine quantitatively the interaction of anti-peptide antisera with the hGR, titration analysis of each antiserum with monomeric GR was performed (Figure 3). [^{3}H]TA-bound hGR, prepared as before, was incubated with various amounts (0.1 µl, 1.0 µl, 10 µl) of each antiserum under high salt conditions (0.5 M NaCl). The antisera used for these studies were from bleeds taken during collection of post-immunization sera. The activities of the anti-peptide

Figure 3. Titration of Anti-hGR Activity.

[^{3}H]TA-labeled hGR was incubated with various amounts of each collection phase antiserum under high salt conditions. The activities of the anti-peptide antisera are expressed as the percentage of counts adsorbed by a 1:5 dilution (10 µl) of AC40. All three anti-peptide antisera are of equal or greater activity than antiserum AC40.


200
100
0
% of AC40
709
710
711
1:500
1:50
1:5
Serum Dilution

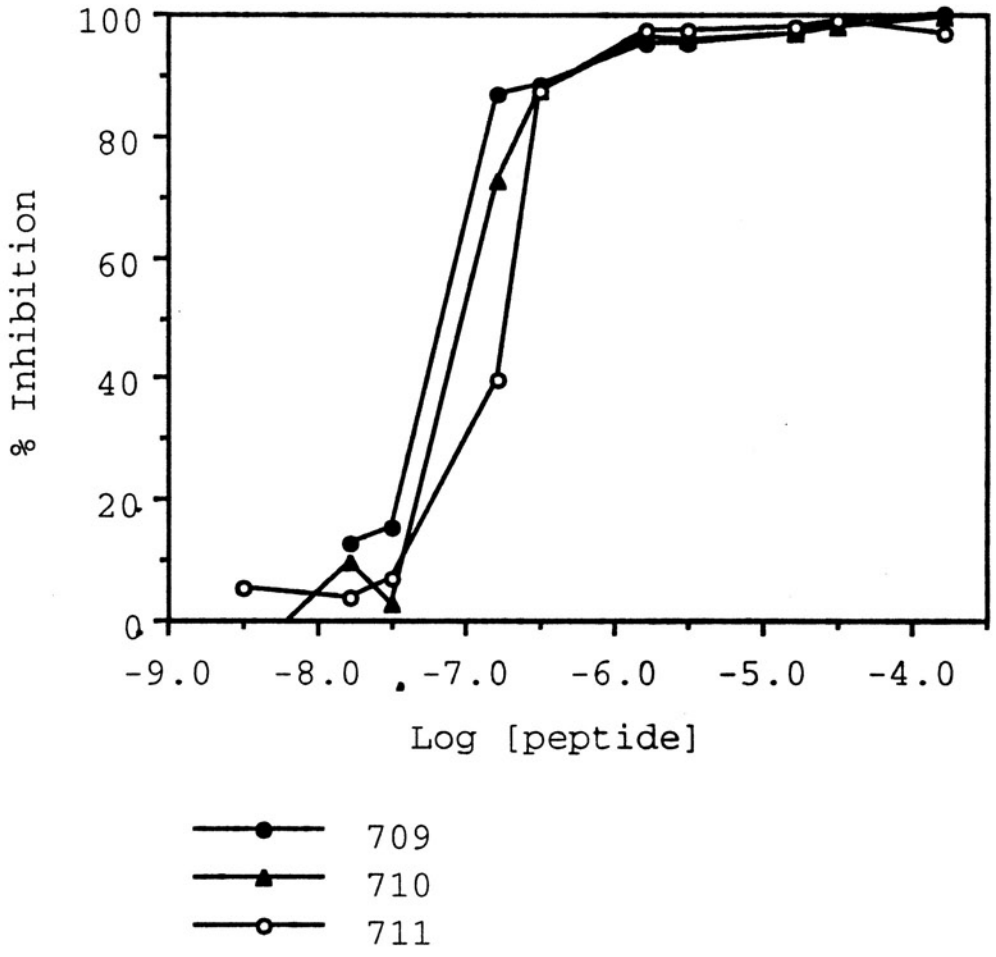
antisera were expressed as the percentage of counts adsorbed by a 1:5 dilution of the antibody AC40. All three antisera were as reactive at a 1:50 dilution as was AC40 at a dilution of 1:5. Thus all three appeared to have a significantly higher titer of anti-hGR antibodies than AC40. In addition, all three antibodies appeared to be similar in titer to each other. Although anti-hGR immunoreactivity was readily observed in Western blots, even at dilutions greater than 1:1000 (data shown below), much higher concentrations of all three antisera were required in immunoadsorption assays to adsorb maximum amounts of receptor. The relatively poor reactivity of these antisera with the native GR suggests that the epitope in the native receptor may not be fully accessible. Alternatively, in the native receptor the conformation of the epitope may have a lower affinity for the anti-hGR antibodies.

2. Competition for antigenic determinants using synthetic peptide.

To confirm the specificity of the antibody-GR interaction, antisera were preadsorbed with free peptide before reaction with the GR (Figure 4). [^{3}H]TA-labeled hGR was incubated with 1.0 µl of each anti-peptide antiserum in the presence of various concentrations (from 10^{-8} to 10^{-4} M) of the immunizing peptide Cys_{245}-Thr_{272}. After adsorption with

Figure 4. Competition for Antigenic Determinants Using Immunizing Peptide.

[^{3}H]TA-labeled hGR was incubated with 1.0 µl (1:50 dilution) of antisera 709(l), 710(s), or 711(m) in the presence of various concentrations (from 10^{-8} to 10^{-4} M) of the peptide cys_{245}-thr_{272}. After adsorption with immobilized Protein A, bound radioactivity was determined as described in "Materials and Methods". Immunoabsorption with antisera was 50% inhibited by the addition of 10^{-7} M Cys_{245}-Thr_{272}; complete inhibition required 10^{-6} M peptide. All three antisera interact with the native hGR specifically with region 245-272, and have approximately the same affinity for the antigenic site.


100
80
60
40
20
0
% Inhibition
-9.0
-8.0
-7.0
-6.0
-5.0
-4.0
Log [peptide]
709
710
711

immobilized Protein A, bound radioactivity was determined. Immunoadsorption with all three antisera was 50% inhibited by the addition of 10^{-7} M Cys_{245}-Thr_{272}, while complete inhibition was observed at 10^{-6} M. These results indicate that all three antisera interact with the native hGR specifically within region 245-272 of the receptor, and have approximately the same affinity for the antigenic site.

3. Interactions between anti-peptide antibodies with native human GR (Sucrose density gradient analysis).

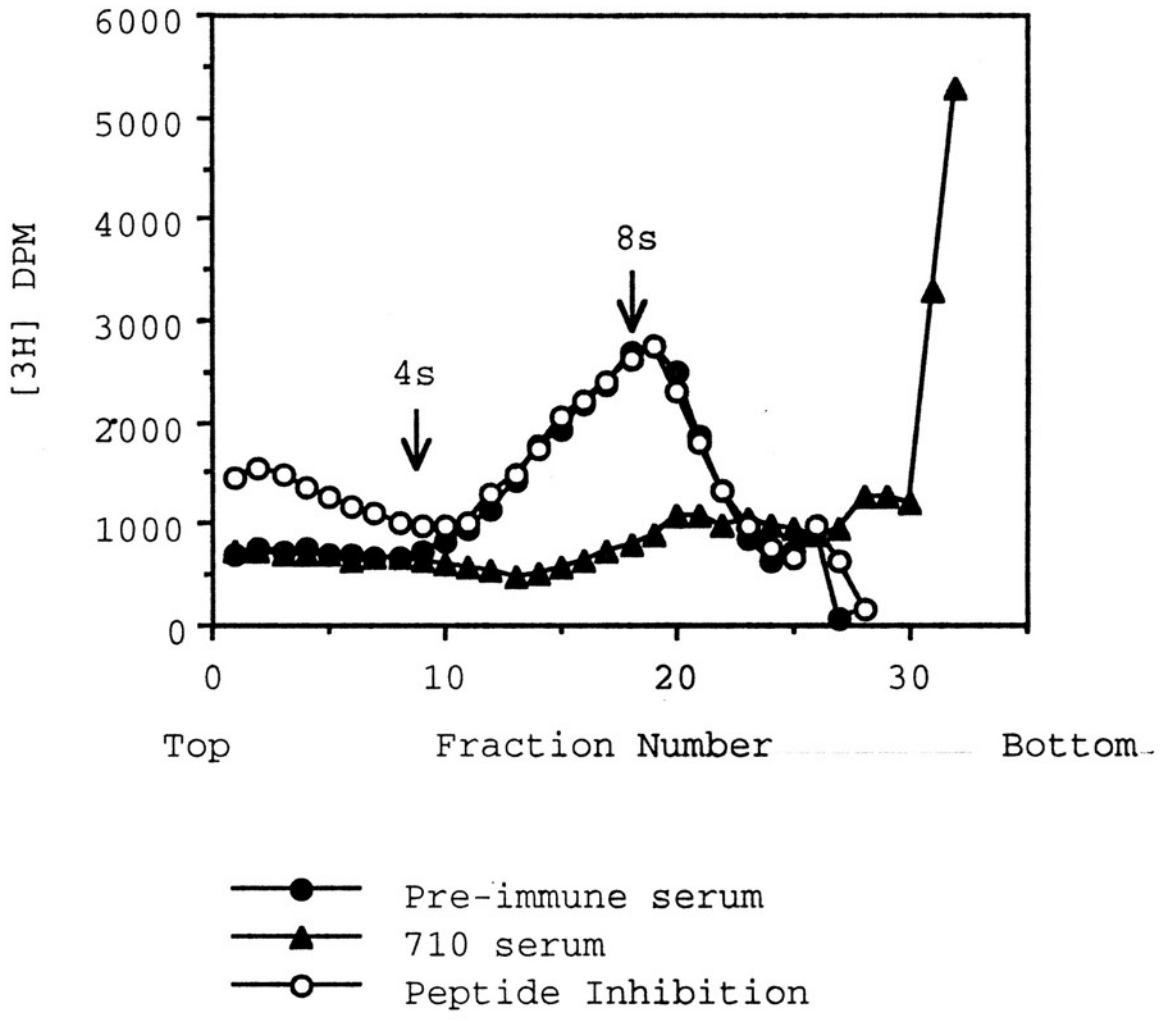
Previous work from our laboratory showed that an antibody raised against a peptide corresponding to a sequence in the hinge region of the hGR recognized only the activated form of the receptor (Urda et al., 1989). We therefore inquired whether the new antisera could recognize both the unactivated and activated forms of the hGR. To investigate the nature of the interaction between these antisera and human glucocorticoid receptor, we employed sucrose density gradient analysis of receptor-antibody complexes. The reactivity of each antiserum with the unactivated 8S glucocorticoid receptor at low ionic strength, and the activated 4S receptor at high ionic strength was examined by assessing the ability of anti-peptide antisera to alter the sedimentation rate of [^{3}H]TA-bound GR.

1). Interaction of anti-peptide antibodies with the unactivated form of human GR.

[^{3}H]TA-GR complexes were incubated with preimmune serum, immune serum, or immune serum preincubated with the immunizing peptide, in the presence of molybdate, an inhibitor of receptor activation which stabilizes the interaction of the steroid binding protein with hsp90. 200 µl samples were layered onto 5-20%, low salt (0.05 M NaCl) sucrose gradients containing 20 mM molybdate. After centrifugation, fractions were collected and analyzed for radioactivity (Figure 5). [^{14}C]-labeled bovine serum albumin ([^{14}C]BSA), which sediments at 3.8S, was included in each gradient as an internal standard. When incubated with preimmune serum, the sedimentation coefficient of the [^{3}H]TA-hGR complex was approximately 8S, the position characteristic of unactivated oligomeric complexes, which have been reported to contain two molecules hsp90 and other components in addition to the steroid-binding protein (Housley et al., 1985; Mendel et al., 1986; Denis et al., 1987; Nemota et al., 1987; Howard et al., 1988). However, when [^{3}H]TA-hGR was incubated with immune serum prior to centrifugation, the resulting radioactive complex displayed a profound increase in sedimentation value from 8S to the bottom of the gradient. This indicated a direct interaction between the molybdate-stabilized GR oligomer and the anti-peptide antibody. The ability of the immunizing peptide to block the interaction of

Figure 5. Interaction of Antipeptide Antibodies With the Unactivated Form of the hGR (Sucrose Gradient).

[^{3}H]TA-hGR complexes (200 µl) were incubated with (10 µl) 710 preimmune serum (l), immune serum 710 (s), or immune serum 710 preincubated with the immunizing peptide (m) in the presence of molybdate. 200 µl samples were layered onto 5-20%, low salt (0.05 M NaCl) sucrose gradients containing 20 mM molybdate. After centrifugation fractions were collected and analyzed for radioactivity. [^{14}C]-labeled bovine serum albumin ([^{14}C]BSA) was included in each gradient as an internal standard. The arrows show 4S and 8S positions respectively. 4S size was determined by the peak of [^{14}C]BSA eluted from the gradient. When incubated with preimmune serum, the [^{3}H]TA-hGR sedimented at approximately 8S, the position characteristic of the unactivated hGR. When [^{3}H]TA-hGR was incubated with immune serum prior to centrifugation, the resulting complex sedimented to the bottom of the gradient. Preincubation of the antibody with the peptide cys_{245}-thr_{272} competitively blocked the effect of the immune serum.


6000
5000
4000
3000
2000
1000
0
[3H] DPM
4s
8s
0
10
20
30
Top
Fraction Number
Bottom
Pre-immune serum
710 serum
Peptide Inhibition

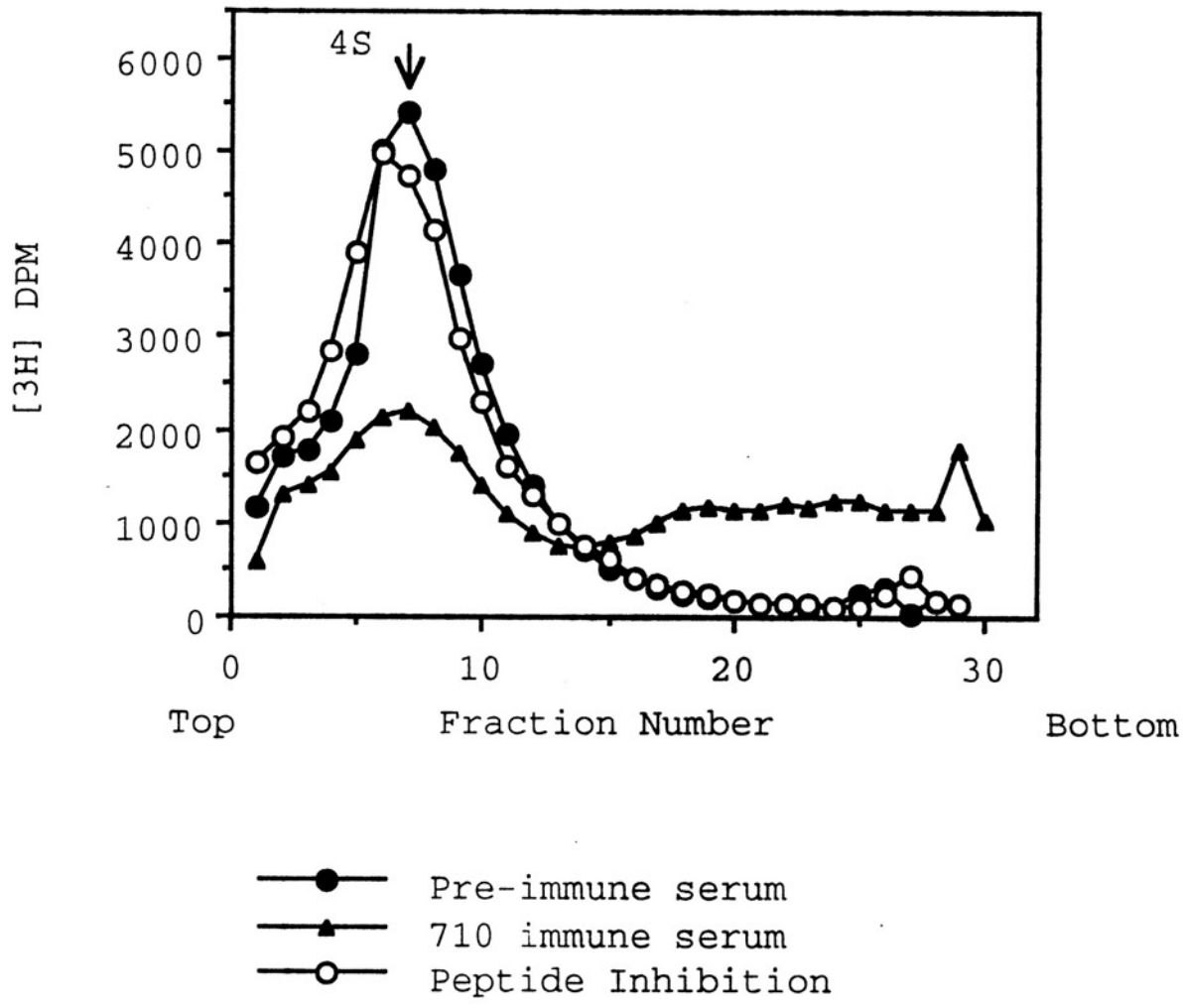
antisera with [^{3}H]TA-bound GR complexes was also investigated. Preincubation of the antibody with the peptide Cys_{245}-Thr_{272} competitively blocked the shift in sedimentation rate caused by the immune sera, and the peak returned to 8S, thus demonstrating the specificity of the antibody-receptor interaction

2). Interaction of anti-peptide antibodies with activated (DNA-binding) forms of human GR.

To examine the interaction of the anti-peptide antisera with the activated form of the hGR, [^{3}H]TA-GR was adsorbed with antiserum in buffer containing high salt (0.5 M NaCl), which promotes receptor activation to the monomeric, 4S form. In addition, molybdate was omitted. The [^{3}H]TA labeled GR preparation was activated at 23^{0}C for 30 minutes prior to immunoadsorption to insure that the interaction of the antibody with receptor was directly with the steroid-binding protein and not with any associated components. The complexes were analyzed on high salt (0.5 M NaCl) sucrose gradients (Figure 6). When [^{3}H]TA-GR complexes were subjected to sucrose density gradient centrifugation in the presence of pre-immune serum, a 3.8-4S peak of radioactivity was observed. The 4S monomeric form of steroid-GR is characteristic of activated complexes, that bind to DNA (Holbrook et al., 1983; Sherman et al., 1983; Vedeckis et al., 1983; Raaka et al., 1985). However, when [^{3}H]TA-GR

Figure 6. Interaction of Antipeptide Antibodies with Activated (DNA-binding) Form of Human GR.

Samples were prepared as in Fig. 5 except that molybdate was omitted from the buffer. The complexes were analyzed on high salt (0.5 M NaCl) sucrose gradients. In the presence of pre-immune serum, a 3.8-4S peak of radioactivity was observed. In the presence of antibody, the complexes appeared to sediment more rapidly as a heterodisperse population. Inclusion of the peptide cys_{245}-thr_{272} during incubation with the antibody competively blocked the antibody-induced shift in sedimentation.


4S
6000
5000
4000
3000
2000
1000
0
[3H] DPM
0
10
20
30
Top
Fraction Number
Bottom
Pre-immune serum
710 immune serum
Peptide Inhibition

complexes were incubated with immune serum prior to centrifugation, the resulting complexes sedimented more rapidly as a complex heterodisperse population. These results indicate a direct interaction between the activated protein and a heterogeneous population of antibodies under high salt conditions. It is interesting to note that a small portion of the activated form is not shifted after incubation with antiserum, suggesting that a small fraction of activated receptor may contain an epitope(s) that is occluded from the antiserum. The mechanism responsible for this effect has not yet been determined. Alternatively, the activated preparation may contain a small amount of proteolyzed receptor which has lost the antibody binding site. In either case, inclusion of the peptide Cys_{245}-Thr_{272} during incubation with the antibody blocked the shift of the 4S receptor to a larger form, verifying the specificity of the interaction of the antiserum with the predicted site in the receptor.

Thus, by several independent criteria, it is clear that the anti-peptide antisera described here recognize both forms of the intact native hGR.

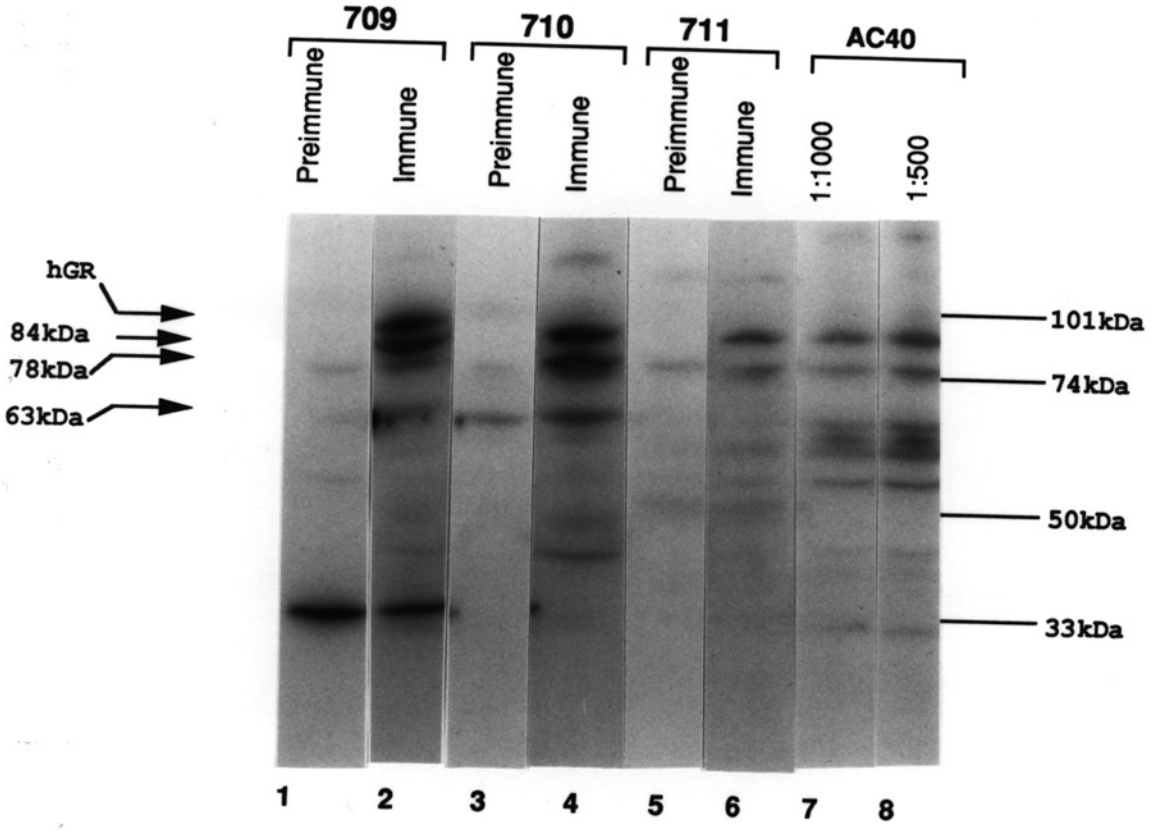
III. Specificity of the anti-peptide antibody for denatured hGR (Western Blot analysis).

To examine the interaction of the anti-peptide antisera with denatured, unliganded GR, IM-9 cell GR was

fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 7). After transfer to nitrocellulose membranes, blots were probed with a 1:1000 dilution of each antiserum, and [^{125}I] protein A. The antisera used were preimmune sera, anti-peptide antisera, and AC40. AC40 antibody was used at dilutions of 1:500 and 1:1000 (lanes 7,8) for purposes of comparison to the anti-peptide antibodies. As shown in Figure 7, a faint band at 92 kDa, comigrating with authentic hGR, was identified using all three immune sera (lanes 2,4,6), but not with pre-immune antiserum (lanes 1,3,5). The molecular weight of 92 kDa is identical to that of hGR resolved with antibody AC40. The major 78 kDa protein band mostly likely represents a truncated receptor synthesized from an internal translation start site. However, additional lower molecular weight bands were also resolved. It is possible that these minor components represent small amounts of proteolyzed receptor, or they may represent interaction of the anti-peptide antisera with other non-receptor proteins. Even though there were few non-specific bands, the sera are still more specific for the hGR than is AC40 (Figure 7). In addition, After improvement of the conditions for washing the nitrocellulose membrane, only 2 major components (Mr=92,000, 78,000) could be identified (Powers and Harmon, unpublished results). Unexpectedly, a 80-90 kDa protein was recognized by antiserum 709. Since this band was not seen with pre-immune serum, its presence may reflect the generation of other non-receptor

Figure 7. Western Blot Analysis of specificity of the Anti-peptide Antibodies for Denatured hGR.

IM-9 cell cytosol was fractionated by polyacrylamide gel electrophoresis and transfered to nitrocellulose membranes. The membranes were probed using a 1:1000 dilution of preimmune sera or immune serum from each rabbit, or AC40. Antibody-receptor complexes were visualized using ^{125}I-protein A and autoradiography. A 92 kDa protein, comigrating with authentic hGR, was identified using all three immune sera, but not with pre-immune antiserum. However, additional lower molecular weight bands were also resolved. These may represent proteolytic receptor fragments. The arrows indicate the position of the intact, 92 kDa human GR, and potential proteolytic receptor fragments. Antibody AC40 was used at dilutions of 1:500 and 1:1000 for purposes of comparison used to identify the 92 kDa receptor protein..


709
710
711
AC40
Preimmune
Immune
Preimmune
Immune
Preimmune
Immune
1:1000
1:500
hGR
84kDa
78kDa
63kDa
101kDa
74kDa
50kDa
33kDa
1
2
3
4
5
6
7
8

antibodies during the production of immune serum. The high molecular weight protein seen only in the AC40 blot is of unknown identity. Since antiserum AC40 was raised against partially purified protein, it is probable that this component is not receptor related. As noted above, the dilutions of anti-peptide antisera suitable for Western blot analysis (1:1000) were considerably greater than required for interaction of these sera with the native receptor. Thus, these sera may have greater affinity for the denatured form of the protein.

DISCUSSION

In the present study, we have described the production and characterization of antibodies against a 28 amino acid peptide which corresponds to residues in the N-terminal region of the hGR. In the past, the majority of antibodies raised against the hGR protein were produced by immunization of rabbits or mice with partially purified receptor preparations. The recent availability of human glucocorticoid receptor cDNA sequences permits the synthesis of peptides based on deduced amino acid sequence. It has become an alternative and useful approach for generating region-specific antibodies to probe the structure and function of steroid receptors.

Several laboratories have recently used synthetic peptides deduced from cDNA sequences in the DNA-binding domain of human glucocorticoid receptor to generate anti-peptide antibodies (Urda et al., 1989; Wilson et al., 1988). These antibodies only recognize the activated, DNA-binding form of the GR protein and provide unequivocal identification of the glucocorticoid receptor in human cell cytosol. However their selective recognition of only the DNA-binding domain limits their utility in the complete analysis of GR function.

The new anti-peptide antibodies we have produced were directed against the poorly conserved amino terminus of the

human GR. They appear to be versatile reagents in that they recognize and interact with the hGR in a variety of physical states. The forms that are identified by the antisera include the liganded-unactivated receptor, the liganded-activated receptor, and the denatured form of the protein. In addition, the molecular weight of the denatured form determined from Western blots using the antipeptide antisera was identical to that obtained using antiserum AC40. Thus, the epitopes against which these antibodies were raised most likely reside on the surface of the GR protein under variety of conditions.

High titer antisera were obtained after three or four immunizations. The antibodies prepared from all three rabbits are IgGs as demonstrated by the ability of protein A to adsorb [^{3}H]TA-receptor complexes. In addition, the similar time courses of immunization for the three rabbits suggest that the production of antibody in response to immunization is a reproducible phenomenon. However, none of the three rabbits yielded high-titer serum for reaction with the native forms of the hGR. Dilutions of antisera required to recognize native receptor (1:50) were considerably lower than those required for Western blot analysis of the denatured receptor (1:1000). In most cases, 1 µl (1:50 dilution) of undiluted serum was required for maximum precipitation of the receptor in 50 µl crude cytosol. This was probably the result of the relative inaccessibly of the native receptor protein to the antibody.

Nevertheless, immune IgG was capable of interacting directly with the liganded glucocorticoid receptor to produce a shift of both activated and unactivated [^{3}H]TA-receptor complexes on sucrose density gradients. Of particular importance is the ability of anti-human receptor antibodies to selectively identify glucocorticoid receptors.

It is interesting to note that the antisera still appear to weakly recognize components with molecular weights of 84 kDa and 64 kDa. At this point, we cannot determine whether these components are proteolytic fragments derived from the 92 kDa receptor or whether they represent other non-receptor related proteins. But compared with the polyclonal antibody AC40, immunoblots revealed fewer nonspecific bands. Furthermore, after improving the membrane washing conditions, only 2 components (Mr=92,000 and 78,000) reacted with immune serum (Powers and Harmon, unpublished results).

All of the data are consistent with the conclusion that the antibodies generated against the synthetic peptide are capable of providing unequivocal identification of the human glucocorticoid receptor. Thus, these anti-peptide anti-human glucocorticoid receptor antibodies provide an extremely powerful tool for the analysis of human glucocorticoid receptors.

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cloning, sequencing and expression of wild-type and mutant receptor proteins. EMBO J. 5: 2513-2522.

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